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FOREWORD

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5. INTRODUCTION

This Annual Report was prepared as a requirement of the Idea Award entitled "Adipocyte Differentiation: Relationship to Breast Cancer" funded by the US Army Medical Research and Materiel Command Breast Cancer Research Program (DAMD17-97-1-7025). This report covers research for the period from 1/1/2000 until 12/31/2000. We will initially provide an overview of this research work. Then, details will be provided in **6. BODY**. The long term objective of this project is to characterize the cellular and molecular mechanisms responsible for intra- and peritumoral accumulation of stromal fibroblasts. To achieve this objective, the following specific aims were proposed. The first **specific aim 1 is to determine whether secretory products of breast cancer cells prevent differentiation of adipose fibroblasts into mature adipocytes**. Major goals in this aim have been accomplished and detailed in the previous annual reports. **Specific Aim 2 is to characterize the secretory products of malignant epithelial cells, which downregulate adipogenic transcription factors**. This aim has also been accomplished completely and results were detailed in the previous annual report. **Specific Aim 3 is to determine whether adipogenic transcription factors regulate aromatase P450 expression in human adipose fibroblasts**. This annual report summarizes primarily the studies outlined in Specific Aim 3 (see 6. BODY). **Specific Aim 4 is to determine the regional distribution of C/EBP β , PPAR γ , C/EBP α and P450arom expression in the breast in relation to tumor location**. During the previous report periods, we characterized the distribution of C/EBP α , C/EBP β and C/EBP γ in adipose tissue fibroblasts proximal and distal to malignant epithelial cells in human breast specimens bearing tumors. As reported previously C/EBP β expression correlates with P450arom expression. We also determined that the malignant epithelial cells is the primary source of TNF α and IL-11 human breast tumor specimens. Thus studies in this aim have also been accomplished.

6. BODY

Expression of aromatase P450 (P450arom), which catalyzes the formation of estrogens, is aberrantly increased in adipose tissue fibroblasts surrounding breast carcinomas giving rise to proliferation of malignant cells. Aromatase in human adipose tissue is expressed primarily in undifferentiated fibroblasts under the control of distinct and alternatively used P450arom promoters. In tumor-free breast adipose tissue, P450arom is expressed at low levels via a distal promoter (I.4), whereas in the breast adipose tissue bearing a tumor, P450arom is increased through the activation of two proximal promoters, II and I.3. Since the *in vivo* activation of P450arom promoter II is a key event responsible for aberrantly high P450arom expression in breast tumors, we studied the molecular basis for the enhancement of P450arom promoter II using human adipose tissue fibroblasts (HAF) in primary culture treated with T47D or breast cancer cell-conditioned medium (TCM) as a model system. Upon treatment by TCM, HAF displayed a striking induction of P450arom mRNA levels via promoter II usage (Figures 1A and B in APPENDIX). This effect appeared to be specific for malignant breast epithelial cells, because conditioned media from breast cancer cell lines T47D and MCF-7 induced promoter II activity, whereas normal breast epithelial cells or liver or prostate cancer cell lines did not produce such an effect. Although treatment with a cAMP analog also caused a switch in the promoter use from I.4 to II in cultured HAF, TCM-induced promoter II use was found to be mediated via a cAMP-independent pathway (Figures 2A and B in APPENDIX). Use of serial deletion mutants of the promoter II 5'-flanking sequence revealed the presence of critical *cis*-acting elements within the -517/-278 bp region, which regulate the baseline and TCM-induced activities (Figure 3 in APPENDIX). TCM caused a 5.7-fold induction of the -517 bp/promoter II construct, whereas site directed mutagenesis of a CCAAT/enhancer binding protein (C/EBP) binding site (-317/-304 bp) abolished both baseline and TCM-induced activities (Figures 4 and 5 in APPENDIX). Ectopic expressions of C/EBP β and to a lesser extent C/EBP α , but not C/EBP, induced promoter II activity (Figure 6 in APPENDIX). Effects of TCM and C/EBP β were not found to be additive on promoter II activity (Figure 7B in APPENDIX). Moreover, we demonstrated the presence of both C/EBP β and C/EBP α but not C/EBP δ in a DNA-protein complex formed by the nuclear extract from TCM-treated HAF and a probe containing the critical C/EBP binding element (-317/-304 bp) (Figure 7A in APPENDIX). Finally, treatment of HAF with TCM strikingly induced C/EBP β mRNA levels, whereas this did not affect the levels of C/EBP α or C/EBP δ mRNA, as determined by northern analysis (Figure 8 in APPENDIX). In conclusion, malignant breast epithelial cells secrete factors, which induce P450arom expression in adipose tissue fibroblasts via promoter II. This is, at least in part, mediated by malignant epithelial cell-induced upregulation of C/EBP β and enhanced binding of this transcription factor to a critical regulatory element (-317/-304 bp) upstream of promoter II.

7. KEY RESEARCH ACCOMPLISHMENTS

- We report hereby a segment of a complex epithelial-stromal interaction between malignant breast epithelial cells and surrounding adipose fibroblasts (preadipocytes) to develop a model whereby malignant cells maximize the number of surrounding undifferentiated adipose fibroblasts and stimulate estrogen production in these fibroblasts.
- Malignant breast epithelial cells stimulate the expression of C/EBP β in adjacent adipose tissue fibroblasts.
- Binding of C/EBP β to aromatase promoter II activates this promoter and switches the promoter use from the physiologically used promoter I.4 to II in adipose fibroblasts treated with cancer cell-conditioned medium. This ultimately gives rise to a striking increase in total aromatase mRNA levels.
- Our previous in vivo data also confirm that promoter II is preferentially activated in adipose tissue adjacent to breast tumors. Thus, our current findings provided a molecular basis for this observation.

8. REPORTABLE OUTCOMES

The following manuscripts bear the acknowledgement of this grant:

1. Meng L, J Zhou, H Sasano, T Suzuki, K Zeitoun, and **SE Bulun**: TNF α and IL-11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively downregulating C/EBP α and PPAR γ : mechanism of desmoplastic reaction. *Cancer Research*. In Press (March, 2001).
2. Zhou J, B Gurates, S Yang, S Sebastian and **SE Bulun**: Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by C/EBP β . *Cancer Research*. In Press (March, 2001).

9. CONCLUSIONS

Our results generated during this report period supported the central hypothesis in this grant application. In summary, we have a two-hit hypothesis. First, malignant epithelial cells block the differentiation of surrounding adipose fibroblasts through cytokines. Then, epithelial factors induce aromatase expression in these undifferentiated fibroblasts via switching aromatase gene promoter use from the physiologically used promoter I.4 to aberrantly activated promoter II. During this grant period, we demonstrated that the transcription factor C/EBP β mediates this malignant epithelial cell effect on adipose fibroblasts. The end result is aromatase overexpression and increased local formation of estrogen in breast cancer. In future, we seek to isolate these malignant epithelial cell-derived factors and associated signaling pathways in adipose fibroblasts. We also will determine whether estrogen regulates the formation of antiadipogenic cytokines IL-11 and TNF α in malignant epithelial cells.

10. REFERENCES

Please refer to references provided in the attached manuscript (APPENDIX).

11. APPENDIX

One appendix that represents the manuscript, which is in press in Cancer Research (see Reportable Outcomes) is attached.

Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by C/EBP β

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(Running title: C/EBP β -mediated upregulation of aromatase by breast cancer)

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ABSTRACT

Expression of aromatase P450 (P450arom), which catalyzes the formation of estrogens, is aberrantly increased in adipose fibroblasts surrounding breast carcinomas giving rise to proliferation of malignant cells. Aromatase in human adipose tissue is primarily expressed in undifferentiated fibroblasts under the control of several distinct and alternatively used P450arom promoters. In tumor-free breast adipose tissue, P450arom is usually expressed at low levels via a distal promoter (I.4), whereas in the breast adipose tissue bearing a tumor, P450arom is increased through the activation of two proximal promoters, II and I.3. Since the *in vivo* activation of P450arom promoter II is a key event responsible for aberrantly high P450arom expression in breast tumors, we studied the molecular basis for the enhancement of P450arom promoter II using human adipose fibroblasts (HAF) in primary culture treated with T47D breast cancer cell conditioned medium (TCM) as a model system. Upon treatment by TCM, HAF displayed a striking induction of P450arom mRNA levels via promoter II usage. This effect appeared to be specific for malignant breast epithelial cells, because conditioned media from breast cancer cell lines T47D and MCF-7 induced promoter II activity, whereas normal breast epithelial cells or liver or prostate cancer cell lines did not produce such an effect. Although treatment with a cAMP analog also caused a switch in the promoter use from I.4 to II in cultured HAF, TCM induced promoter II use was found to be mediated via a cAMP-independent pathway. Use of serial deletion mutants of the promoter II 5'-flanking sequence revealed the presence of critical cis-acting elements in the -517/-218 bp region, which regulate the baseline activity. TCM caused a 5.7-fold induction of the -517 bp promoter II construct, whereas site directed mutagenesis of a CCAAT/enhancer

binding protein (C/EBP) binding site (-317/-304 bp) abolished both baseline and TCM-induced activities. Ectopic expressions of C/EBP α and C/EBP β , but not C/EBP δ , significantly induced promoter II activity. Moreover, we demonstrated the presence of both C/EBP β and C/EBP δ but not C/EBP α in a DNA-protein complex formed by the nuclear extract from TCM-treated HAF and a probe containing this critical C/EBP binding element (-317/-304 bp). Finally, treatment of HAF with TCM strikingly induced C/EBP β expression, whereas this did not affect the levels of C/EBP α or C/EBP δ transcripts. In conclusion, malignant breast epithelial cells secrete factors, which induce aromatase expression in adipose fibroblasts via promoter II. This is, at least in part, mediated by a TCM-induced upregulation and enhanced binding of C/EBP β to a promoter II regulatory element.

INTRODUCTION

The conversion of C₁₉ steroids to estrogens by aromatase P450 (P450arom) takes place in a number of human cells, *e.g.*, the ovarian granulosa cell (1), skin and adipose fibroblasts (2, 3). Aromatase expression in the adipose tissue is limited to fibroblasts and is not detected in significant quantities in the fully differentiated and lipid-filled adipocytes (2, 3). Aromatase activity in adipose fibroblasts has long been implicated in the pathophysiology of breast cancer growth (4-7). Estrogen produced in breast adipose tissue acts locally to promote the growth of tumor (8). Thus, the relationship between adipose stroma and breast cancer is unique in that the adipose fibroblast provides structural and functional support for cancer growth. O'Neill and coworkers demonstrated that the breast quadrant displaying the highest level of aromatase activity was consistently involved with tumor (6). Subsequently, we found the highest levels of P450arom transcripts in adipose tissue from the quadrant bearing a tumor (7). In the same study, tumor-bearing quadrants contained the highest fibroblast-to-adipocyte ratios. It follows then that the breast quadrant with the highest fibroblast content contains the highest levels of P450arom transcripts. The clinical relevance of these observations has been exemplified by the successful treatment of breast carcinomas with potent aromatase inhibitors (9-11).

Expression of the human P450arom (*CYP19*) gene is under the control of several distinct and partly tissue-specific promoters (12, 13). Three of these promoters (I.4, I.3 and II) are used in adipose tissue. Interestingly, in disease-free breast adipose tissue, P450arom is usually expressed at low levels via a distal promoter (I.4), whereas in the adipose tissue of the breast bearing a tumor, P450arom expression is increased through

the activation of two proximal promoters II and I.3 (14, 15, 16). In addition to these *in vivo* observations, treatments of human adipose fibroblasts in culture with various hormones switch promoter use. For example, glucocorticoids plus cytokines induce P450_{arom} expression via promoter I.4 in cultured primary human breast adipose fibroblasts (HAF), whereas treatment with a cAMP analogue switches the promoter use to II and I.3 (12, 13). We hypothesize that malignant breast epithelial cells interact with the surrounding adipose tissue fibroblasts to activate promoters II and I.3. The data presented in this report will serve to reconcile the *in vivo* and *in vitro* observations summarized above (12,13, 14). We report a novel epithelial-stromal interaction, which favors the induction of P450_{arom} expression in HAF by malignant epithelial cells via promoter II.

We and others have previously shown that breast cancer cells could stimulate aromatase expression in HAF, which was suggestive of a cross-talk between malignant epithelial cells and surrounding HAF to favor estrogen production in breast tumors (17-19). We recently demonstrated that medium conditioned with malignant epithelial cells inhibited the differentiation of HAF to mature adipocytes via the suppression of the essential adipogenic transcription factors CCAAT/enhancer binding protein (C/EBP) α and peroxisome proliferator activated receptor (PPAR) γ . C/EBP β and C/EBP δ , on the other hand, were upregulated in these undifferentiated murine fibroblasts treated with T47D cell-conditioned medium (TCM) (Meng, et al, unpublished data). TCM-induced decreases in C/EBP α or PPAR γ were sufficient to completely inhibit adipogenic differentiation of 3T3-L1 cells in our hands (Meng, et al, unpublished data). This was in agreement with previously published reports (20, 21). On the other hand, we were intrigued by the TCM-induced increases in C/EBP β and C/EBP δ mRNA levels in 3T3-

L1 murine cells (Meng, L., et al, unpublished data). In contrast to C/EBP α or PPAR γ , ectopic expressions of C/EBP β or C/EBP δ were not sufficient to induce adipocyte differentiation in the absence of C/EBP α or PPAR γ (22). Thus, we hypothesize that breast cancer-induced increases in C/EBP β or C/EBP δ levels do not affect adipocyte differentiation but may serve to increase aromatase expression in adipose fibroblasts surrounding the cancer. We used herein a model whereby human T47D breast cancer cell conditioned medium (TCM) is added to primary human breast adipose fibroblasts (HAF) to understand the roles of C/EBP isoforms in the upregulation of aromatase expression in undifferentiated fibroblasts. We chose to study the activation of promoter II, since work from three different laboratories demonstrated that the activity of this promoter was upregulated *in vivo* in breast stroma bearing a carcinoma (14, 15, 16).

MATERIALS AND METHODS

Cell cultures

Human adipose tissues were obtained at the time of surgery from women undergoing reduction mammoplasty following a protocol approved by the Institutional Review Board for Human Research of the University of Illinois at Chicago. For primary human adipose fibroblast cultures, adipose tissues were * minced and digested with collagenase B (1 mg/ml) at 37°C for 2 h. Single-cell suspensions were prepared by filtration through a 75 μ m sieve.* Fresh cells were suspended in DMEM/F-12 containing 10% FBS in a humidified atmosphere with 5% CO₂ at 37°C. Twelve to 24 h after the attachment of fibroblasts, culture medium was removed and cell medium was changed at 48h intervals until the cells became confluent. Before total RNA or nuclear proteins were extracted from HAF, these cells were cultured in either serum-free DMEM/F-12, DMEM/F-12

containing 10% FBS, serum-free DMEM/F-12 containing dibutyryl cyclic adenosine monophosphate (Bt₂cAMP, 0.5 mM) together with phorbol diacetate (PDA, 100 nM), DMEM/F-12 containing 10% FBS plus DEX (250 nM) or DMEM/F-12 conditioned with malignant or benign * cells. All treatments were continued for 48 h.

T47D cells purchased from American Type Culture Collection (ATCC, Rockville, MD) were initially grown in RPMI 1640 with 10% FBS containing 0.02 mM Hepes, whereas MCF-7 cells, prostate cancer cell line PC-3 and hepatocellular carcinoma cell line HepG2 (ATCC, Rockville, MD) were grown in MEM with 10% FBS. Human normal mammary epithelial cells (HMEC) purchased from Clonetics Inc. (Walkersville, MD) were grown in fully supplemented MEGM medium (Clonetics, Walkersville, MD). Before shipment, these cells were passed twice and demonstrated to contain immunoreactive cytokeratins 14 and 18. In our hands, these cells were alive and dividing every 48 to 72 h. Conditioned media from T47D (TCM), MCF-7, PC-3, HepG2 or HMEC cells (NCM) were collected to be used subsequently as treatments on HAF. To collect conditioned media, cells were initially grown to confluence and switched to DMEM/F-12 for a 12 h of washout period, then, cells were incubated in DMEM/F-12 for 24 hours to allow accumulation of secreted factors in the medium. *

Reverse Transcription-Polymerase Chain Reaction Amplification (RT-PCR)

Amplification of the untranslated 5'-ends of P450arom transcripts from HAF under various treatments was accomplished with exon-specific oligonucleotide pairs as described below. * Five microgram of DNase I-treated total RNA was used for RT reaction. Five microliters of RT mixture was amplified using PCR.* For the amplification of total P450arom transcripts, 5'-end sense primer from coding exon II (5'-

TTG GAA ATG CTG AAC CCG AT-3') and 3'-end antisense primer complimentary to coding exon III (5'-CAG GAA TCT GCC CTG GGG AT-3') were used. To amplify promoter-specific 5'-untranslated sequences, primers for promoter II-specific sequence (5'-GCA ACA GGA GCT ATA GAT-3') and exon I.4 (5'- GTA GAA CGT GAC CAA CTG G -3') were used as 5'-end sense primers together with an antisense primer complimentary to the coding exon III (5'-ATT CCC ATG CAG TAG CCA GG -3'). PCR conditions were as the following: denaturing at 95°C for 30 s, annealing at 55 °C for amplification of promoter II-specific sequence or 58°C for amplification of exon I.4 and the coding region for 40 s, and extension at 72°C for 40 s for 30 cycles. * Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as an endogenous marker to check the integrity of cDNA. A 5'- end sense primer (5'- CGG AGT CAA CGG ATT TGG TCG TAT -3') and a 3'- end antisense primer (5'- AGC CTT CTC CAT GGT GGT GAA GAC-3') were used for amplifying a 306 bp-long sequence in GAPDH mRNA. PCR conditions were the same as those used for amplification of promoter II-specific fragments, except for the number of cycles (20) and the quantity of RT mixture (0.5 µl). * This RT-PCR method was described previously in greater detail (14).

Determination of intracellular cyclic AMP

HAF were plated in six-well 35 mm culture dishes. After reaching confluence, HAF were cultured either in serum-free DMEM/F-12, DMEM/F-12 containing 10% FBS, DMEM/F-12 containing 10% FBS and forskolin (10 µM), DMEM/F-12 containing 10% FBS and DEX (250 nM) or T47D cell conditioned DMEM/F-12. Measurements were performed in triplicate replicates and treatments were carried for 0, 12, 24 and 48 h. HAF

were lysed in a 0.1 M HCl solution * after the removal of media. Cell lysis mixture was centrifuged and the supernatant was then used directly in the cAMP assay using Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Design, Inc., Ann Arbor, MI) following the protocol supplied by the vendor. Briefly, 50 μ l of the pink-neutralizing reagent was added into each well, except for the total activity and blank wells. Samples (100 μ l) were then added to appropriate wells. Fifty microliter of the conjugate was added into each well followed by the addition of 50 μ l of the yellow-antibody. After incubating at room temperature for 2 h on a shaker at 500 rpm, the plate was washed three times with 200 μ l of washing buffer followed by the addition of substrate solution 200 μ l to each well. The stop solution (50 μ l) was then added to each well and the optical density was read at 405 nm with correction to 570 nm. Results were obtained by plotting on the standard curve.

Transient transfections and luciferase assays

HAF in primary culture were transfected using Lipofectamine PlusTM (Gibco-BRL, Grand Island, NY) with the following plasmids: (i) 1 μ g of modified PGL₃-Basic Luciferase reporter plasmid that contains serial deletion mutants of P450arom promoter II; (ii) 0.2 μ g of pcDNA3 expression plasmid (Invitrogen, Carlsbad, CA), which contains the cDNA of either C/EBP α (human), C/EBP β (rat) or C/EBP δ (rat); (iii) 5 ng of pRL-CMV renilla Luciferase control reporter vectors that contains the cDNA encoding renilla Luciferase (Promega, Madison, WI) as an internal control for transfection efficiency. The day before transfection, HAF in primary culture were seeded into 35mm-dishes at 2×10^5 cell/dish. * The transfection solution was made of 200 μ l of OPTI-MEM I reduced-serum medium containing PLUS reagent (8 μ l), pre-complexed DNA (1.2 μ g) and 5 μ l of lipofectamine reagent. After transfection for 6 h in transfection solution at 37°C in 5%

CO₂, medium was changed to antibiotic-free DMEM/F-12 containing 10% FBS for overnight recovery. Cells were then switched to media conditioned by normal breast epithelial cells or T47D cells for another 48 h. Luciferase and renilla Luciferase assays were performed using a dual-Luciferase reporter assay system kit (Promega, Madison, WI). * Results are presented as the average of data from triplicate replicates and expressed as the ratio to the internal standard renilla Luciferase. The empty Luciferase vector PGL₃-Basic was arbitrarily assigned a unit of 1, and the rest of the results were expressed as multiples of the PGL₃-Basic vector. *

Northern blotting

Total RNA was isolated from HAF in primary culture growing in (i) DMEM/F-12, (ii) normal breast epithelial cell conditioned medium or (iii) T47D cancer cell conditioned medium (TCM). Twenty micrograms of total RNA used.* Complementary DNA probes for C/EBP δ , C/EBP β and C/EBP α were prepared from plasmids kindly provided by Drs. Steve McKnight, Gokhan Hotamisligil, Carole Mendelson, Bruce Beutler and Gretchen Darlington. *

Site-directed mutagenesis

To generate serial plasmids bearing mutated consensus-binding sequences for transcription factors of C/EBPs, steroidogenic factor-1 (SF-1) and cAMP response element binding protein (CREB), site-directed mutagenesis was performed using the GeneEditor™ *in vitro* site-directed mutagenesis system (Promega, Madison, WI), as the manufacturer's instructions. A -517 bp promoter II/PGL₃-Basic construct containing wild type -517/-16 bp of P450arom promoter II 5'-flanking DNA was used as a template for

site-directed mutagenesis. Briefly, DNA template (0.5 pmol) was denatured and annealed with mutagenic and selection oligonucleotides. Mutant strand was synthesized in the reaction mixture containing 1× synthesis buffer, 5 units of T4 DNA polymerase and 2 units of T4 DNA ligase at 37°C for 90 min. The mutagenesis reaction mixture was then used to transform BMH 71-18 *mutS* competent cells. These transformed competent cells were incubated in a medium containing GeneEditor™ antibiotic selection mix overnight to select the desired mutant plasmids. The plasmids isolated from the BMH 71-18 *mutS* were transformed into JM109 competent cells. The transformed JM109 competent cells were grown overnight on the LB plates containing ampicillin and GeneEditor™ antibiotic selection mix to further select the mutated plasmids. The mutation of binding consensus was confirmed by DNA sequencing. Consensus binding sequences for mutation and primers used were depicted in Table 1.

Electrophoresis mobility shift analysis (EMSA)

The nuclear extracts used for EMSA were prepared as previously described (23). Briefly, cells were grown to confluence and cultured in either DMEM/F-12 only or T47D cell conditioned DMEM/F-12 for 48 h. Cells were then * scraped from the dishes. * The cell pellet was re-suspended in * cold buffer A (10 mM HEPES, pH 7.4; 1.5 mM MgCl₂; 10 mM KCl; 9.5 mM DTT; leupeptin, 10 µg/ml; pepstain, 100 µg/ml, aprotonin, 2 µg; 0.5 mM phenylmethyl-sulfonyl fluoride). The cell pellets were homogenized * on ice. Once greater than 90 % of the cell membranes were broken, the lysate was * centrifuged for 2 min at 700 g. After the supernatant was removed, the nuclear pellet was resuspended in a one * volume of buffer C (20 mM Hepes, pH 7.4; 420 mM NaCl; 1.5 mM MgCl₂; 0.2 mM EDTA; 0.5 mM DTT; 20% glycerol) and incubated on ice for 30 min with

intermittent mixing. After centrifugation at 60,000 rpm for 5 min at 4°C, the supernatant was snap-frozen in liquid nitrogen. Protein concentrations were determined by a modified Bradlord assay (Bio-Rad, Hercules, CA), and nuclear extracts were stored at -80°C.

* Double-stranded oligonucleotides were obtained through annealing sense and anti-sense sequences. The double-stranded oligonucleotide probes were end-labeled with [γ -³²P]ATP using T4 kinase. * EMSAs were performed as previously described (23). Briefly, a 5 μ g of nuclear extract was incubated with the radiolabeled double-stranded oligonucleotide probe for 15 min at room temperature in a reaction buffer containing Hepes (20 mmol/L, pH 7.6), KCl (75 mmol/L), EDTA (0.2 mmol/L), 20% glycerol and 2 μ g of poly(dI-dC)-poly(dI-dC) as a nonspecific competitor. Protein-DNA complexes were resolved on 6% nondenaturing polyacrylamide gels. * EMSAs were performed after the addition of 0.5 μ l of an antibody against C/EBP α , C/EBP β , C/EBP δ or CREB to the binding reaction, followed by a 30-min incubation on ice before electrophoresis. All antibodies were purchased from Santa Cruz (California, CA). We used the following double-stranded probes: C/EBP binding site probe (5'-GAA GAA GAT TGC CTA AAC AA-3') represents an identical 20 bp long sequence (-303/-322) in the promoter II regulatory region of the P450arom gene. Mutated C/EBP binding site probe (5'-GAA GAA Gcc cGC CTg gtC AA-3') contains a mutated version of C/EBP binding motif that does not interact with any of the C/EBP isoforms.

RESULTS

Aromatase expression in human adipose fibroblasts (HAF) is stimulated by breast cancer cell conditioned medium via P450arom promoter II

Aromatase expression in human tissues is under the control of alternatively used and partially tissue-specific promoters. The coding region of P450arom transcripts and, thus, the translated protein, however, are identical in each tissue site of expression. In the breast adipose tissue of disease-free women, P450arom expression is expressed at low levels via a distal promoter I.4, whereas in the adipose tissue of breast bearing a tumor, P450arom expression is increased through activation of an ovarian-type proximal promoter II (14, 15, 16). We attempted to verify these *in vivo* data by the following *in vitro* experiments. Since the use of each alternative promoter gives rise to a P450arom transcript with an untranslated 5'-end unique for that particular promoter, we used exon-specific RT-PCR to determine total and promoter-specific P450arom transcript levels in HAF in primary culture treated with T47D cell conditioned medium (TCM). As expected, Bt₂cAMP plus PDA stimulated P450arom transcript levels primarily via activation of promoter II, whereas dexamethasone (DEX) plus serum activated promoter I.4. Most importantly, we found that TCM stimulated P450arom transcripts levels via P450arom promoter II activation, which was previously demonstrated *in vivo* in adipose tissue of the breast bearing a tumor (Figure 1A). To address the specificity of effects of T47D cells on HAF, we used media conditioned with either MCF-7 breast cancer cell line or normal breast epithelial cells (NCM) to treat HAF. Medium conditioned with MCF-7 cells but not with normal epithelial cells induced P450arom transcript levels via promoter II (Figure 1B). Furthermore, other malignant cell lines HepG2 and PC-3 failed to activate

P450arom transcripts via promoter II, which demonstrated the stimulatory effect produced by T47D and MCF7 cells was specific for breast cancer (Figure 1C). These results demonstrate that malignant breast epithelial cells in culture produce specific factors, which stimulate aromatase expression *via* promoter II.

Activation of P450arom promoter II by breast cancer cell conditioned medium is not cAMP-dependent

Since both TCM and cAMP analogues induce aromatase expression in HAF via promoter II, we sought to determine whether this effect of TCM is mediated via increased formation of cAMP in HAF. Therefore, we first measured the intracellular levels of cAMP in HAF treated with forskolin, fetal bovine serum (FBS), FBS plus dexamethasone (DEX) or TCM. Contrary to our expectations, treatment with TCM decreased intracellular levels of cAMP at 12, 24 and 48 h time points (Fig 2A). FBS or FBS plus DEX also decreased cAMP levels, whereas treatment with the adenylate cyclase inducer forskolin (10 μ m) gave rise to a striking increase in cAMP levels in HAF (positive control) (Fig 2A). On the other hand, addition of the adenylate cyclase inhibitor SQ 22,536 to the culture medium $\frac{1}{2}$ h before the treatment with TCM for 48 h did not inhibit promoter II activation (negative control) (Fig 2B). These results indicate that the activation of P450arom promoter II by TCM is mediated via a cAMP-independent pathway.

Regulation of P450arom promoter II activity in primary human adipose fibroblasts

Next, we determined the genomic regions critical for the regulation of baseline levels of promoter II activity in HAF (Fig 3). Use of serial deletion mutants of promoter II fused

to Luciferase reporter gene demonstrated that the -517/-278 bp region contained critical stimulatory elements, whereas the -694/-517 region contained inhibitory elements for the baseline activity (Fig 3).

The C/EBP binding sequence (-317/-304 bp) is essential for the breast cancer cell induced activation of promoter II

We identified two C/EBP binding sites in the -517/-278 bp region using the TFSEARCH database (<http://www.blast.genome.ad.jp/sit/TFSEARCH>) (Fig 4). The -278/-100 bp region contains 2 SF-1 sites and a CRE. One of these SF-1 binding sites (-136/-124 bp) and CRE (-211/-197) were previously shown to be critical for cAMP-induced promoter II activity in ovarian granulosa cells and endometriosis-derived stromal cells (23, 24). We determined the effect of T47D breast cancer cell conditioned medium (TCM) on the activity of the -517 bp promoter II/Luciferase construct, since this construct showed the highest baseline activity (Fig 3). Treatment with TCM for 48 h induced the activity of -517 bp construct by 5.7 fold (Fig 5). Site directed mutagenesis of 5 potentially important *cis*-acting elements demonstrated that CRE (-211/-197 bp) and a C/EBP binding site (-317/-304 bp) were essential for TCM induction of promoter II activity. In particular, mutation of the -317/-304 bp C/EBP binding site completely abolished both baseline and TCM-induced activities. Mutation of the two SF-1 sites or the -350/-337 bp C/EBP binding site did not effect TCM induction of promoter II activity (Fig 5). We found that medium conditioned with normal breast epithelial cells (NCM) did not change the activity of -517 bp construct in comparison to incubation with DMEM/F-12 only (data not shown). Therefore, promoter II activity in NCM-treated HAF is similar to the

baseline level. In this particular experiment illustrated in Fig 5, we determined the TCM fold induction of promoter in comparison with NCM treatment.

Induction of promoter II activity in HAF by factors derived from T47D breast cancer cells is mediated by C/EBP β

Figure 6 depicts the effects of the adipogenic transcription factors, C/EBP α , C/EBP β and C/EBP δ on the activity of -517 bp promoter II construct in HAF. Ectopic expressions of C/EBP β (3.5-fold) and C/EBP α (2.5-fold) stimulated promoter II activity, whereas C/EBP δ did not have any significant effect (Fig 6).

Thus far, these results were indicative of TCM induction of promoter II activity via a C/EBP binding site (-317/-304 bp). Ectopic expressions of C/EBP α and C/EBP β significantly stimulated the -517 bp promoter II construct (Fig 6). In order to determine whether C/EBP α or C/EBP β mediates TCM induction of promoter II, EMSA was employed using an oligonucleotide probe (-322/303 bp) containing the -317/-304 bp C/EBP binding site, nuclear extracts from HAF incubated with or without TCM and supershifting antibodies against C/EBP α , C/EBP β and C/EBP δ . This C/EBP binding site (-317/-304bp) was chosen to be included in the probe, since this element was found to be critical for TCM activation of promoter II (Fig 5). We identified two specific complexes (1 and 2) as verified by a wild type (WT) and mutated (Mut) cold competitors in TCM-treated HAF (Fig 7). Antibodies against both C/EBP β and C/EBP δ supershifted complex 1 indicating the presence of C/EBP β and C/EBP δ . On the other hand, antibodies against C/EBP α or CREB did not eliminate or supershift any of these complexes. To further directly investigate whether the activation of P450arom promoter II is mediated by C/EBP β , we demonstrated that the effects of TCM and C/EBP β were not additive. TCM

stimulated the -517 construct by 6 fold, whereas the addition of C/EBP β to TCM did not further increase this induction, which was suggestive that the effects of TCM on promoter II were, at least in part, mediated by C/EBP β (Figure 7B).

These experiments were suggestive that TCM-induction of promoter II activity was mediated by C/EBP β but not by C/EBP α or C/EBP δ , because C/EBP α does not bind to the regulatory element at -317/-304 bp, which is critical for TCM stimulation of promoter II. Although C/EBP δ binds to this site, ectopic expression of C/EBP δ does not increase promoter II activity. To confirm this conclusion we determined the effects of TCM on the mRNA levels of C/EBP isoforms in HAF. Treatments with TCM or normal epithelial cell conditioned medium (NCM) did not change the mRNA levels of C/EBP α or C/EBP δ . On the other hand, only TCM induced C/EBP β expression in HAF strikingly (Fig 8). Thus, we conclude that TCM induction of P450arom promoter II in HAF is mediated, at least in part, by the induction of the expression of C/EBP β , which binds to the -317/-304 bp region in this promoter.

DISCUSSION

The understanding of the molecular mechanisms that are responsible for aberrant P450arom expression in tumor-bearing breast adipose tissue may provide insights into the etiology of breast cancer and lead to the identification of molecular targets for the development of novel treatment strategies. Investigators from at least four different laboratories have demonstrated strikingly increased levels of aromatase activity and P450arom mRNA in breast adipose tissue containing a tumor compared with breast tissue from disease-free women (6, 7, 14, 15, 16). It was also consistently found that upregulation of promoter II activity was responsible, in part, for increased aromatase expression in breast cancer (14, 15, 16). Although it was suggested that promoter II upregulation by breast tumors might be mediated by PGE₂ and cAMP, no direct evidence to support this concept has been provided to date (25). Another report, on the other hand, support our findings regarding the effect of MCF-7 breast cancer cells on switching the promoter use from I.4 to II (26). The downstream signal transduction events or the specificity of breast epithelial cell types, however, have not been characterized in this article (26). We herein present data to support that malignant breast cells induce aromatase expression *via* promoter II employing a cAMP-independent mechanism. A key event is the binding of the adipogenic factor C/EBP β to a specific cis-acting element upstream of promoter II to activate its transcription. We have used an *in vitro* system to support our conclusion. The use of malignant and normal breast epithelial cell conditioned media with clear and consistent biological effects on fibroblasts and employment of positive and negative controls for cell types and various components of signal transduction pathways, however, offset the disadvantages of using an *in vitro*

system and permit the performance of useful mechanistic experiments. Additionally, our conclusions are supported by *in vivo* data from human breast cancer specimens, which showed downregulation of C/EBP α but persistent expression of C/EBP β and C/EBP δ proximal to malignant cells (Meng, L., et al, unpublished observations). Based on these data, we suggest the following model: Breast cancer cells secrete cytokines that selectively downregulate essential adipogenic factors, which inhibit the differentiation of fibroblasts to mature adipocytes. Estrogen production in these fibroblasts maintained in the undifferentiated state by malignant cells is further enhanced by tumor-derived factors, which exist in the T47D or MCF-7 cell conditioned media. These factors act via a cAMP-independent pathway to increase C/EBP β expression in adipose fibroblasts and enhance the binding of C/EBP β to a specific promoter II regulatory sequence. The end result is increased local estrogen concentration in the breast tumor .

We do not know yet the identities of the unknown factors in TCM, which increase C/EBP β expression and P450arom promoter II activity in adipose fibroblasts. Cytokines such as TNF α and IL-6 were shown to increase the transcriptional activity of C/EBP β (27, 28). It is not clear, however, whether these cytokines increase C/EBP β expression or promoter II activity. Our preliminary findings and previous publications demonstrated that these cytokines (IL-11, IL-6, and TNF α) do not activate P450arom promoter II, which is upregulated *in vivo* in breast tumors. Instead, these substances activate promoter I.4, which is not upregulated in tumors (17, 29, 30, 31, our unpublished observations). Therefore, these cytokines by themselves probably do not account for the *in vivo* upregulation of aromatase expression in breast tumors. Our efforts will continue to identify these unknown factors originating from malignant cells to induce aromatase expression in the adipose fibroblast via promoter II.

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Figure Legends

Figures 1A and B. Breast cancer cells stimulate P450arom expression via promoter II in human primary adipose fibroblasts (HAF).

HAF in primary culture were incubated under various conditions for 48 h (No treatment: serum-free DMEM/F-12; TCM: T47D cell conditioned medium; DEX + FBS (10%): Dexamethasone (250 nM) and fetal bovine serum; Bt₂cAMP + PDA: 0.5 mM Bt₂cAMP + 100 nM phorbol diacetate; 10% FBS: DMEM/F-12 + 10% FBS; MCF-7-CM: MCF-7 cell conditioned medium; NCM: normal mammary epithelial cell conditioned medium). Total RNA was subjected to exon-specific RT-PCR to amplify promoter-specific untranslated first exons. GAPDH was amplified to control the integrity of RNA. **Panel A:** Total P450arom transcript levels were upregulated by treatments with T47D-CM and Bt₂ cAMP + PDA via the use of promoter II. DEX + FBS treatment, on the other hand, robustly induced P450arom transcripts via another promoter, promoter I.4. **Panel B:** Medium conditioned by another breast cancer cell line, MCF-7, also induced P450arom transcripts *via* promoter II usage, whereas normal epithelial breast cell conditioned medium (NCM) did not induce P450arom transcript levels at all. **Panel C:** Media conditioned by prostate cancer cell line PC-3 and hepatocellular carcinoma cell line HepG2 failed to induce promoter II or the levels of total P450arom transcripts (coding region).

Figures 2A and B. Activation of P450arom promoter II by T47D cell conditioned medium is not cAMP-dependent.

HAF were incubated under various conditions (No treatment: serum-free DMEM/F-12; 10% FBS: DMEM/F-12 + 10% FBS; Forskolin: 10 μ M forskolin; DEX + 10% FBS: 250 nM DEX + 10% FBS; TCM: T47D cell conditioned-medium; NCM: normal mammary epithelial cell conditioned medium; TCM + SQ22,536: T47D cell conditioned medium + 100 μ M SQ 22,536; No RT: no RT reaction mixture for negative control). HAF were then sampled at 0, 12, 24, 48 h for intracellular cAMP assay or at 48 h for semi-quantitative RT-PCR. **Panel 2A:** TCM did not increase the intracellular cAMP levels at 12, 24 and 48h as in treatments with FBS and DEX-FBS. On the other hand, forskolin, an adenylate cyclase inducer, strikingly increased cAMP levels, as expected. **Figure 2B:** SQ 22,536, an adenylate cyclase inhibitor, could not eliminate the TCM-induced induction of P450arom promoter II-specific transcripts.

Figure 3. Regulation of P450arom promoter II activity in primary human adipose fibroblasts

Luciferase plasmids containing the 5'-flanking region of human P450arom promoter II with serial deletions (-100, -140, -214, -278, -517, -694 bp) were transfected into HAF. pCMV renilla was used as an internal control for transfection efficiency. Promoter II activity was normalized to pCMV renilla and was represented as the average of data from triplicate replicates \pm SEM. The empty Luciferase vector PGL3-Basic was arbitrarily assigned a unit of 1, and the rest of the results were expressed as multiples of the PGL3-Basic vector. We conclude that the -517/-278 bp region contains critical stimulatory

elements, whereas the –694/-517 region contains inhibitory elements, which regulate the baseline activity.

Figure 4. Potential cis-acting elements located in the P450arom promoter II region

A computer-assisted search revealed two C/EBP binding sites at -350/-337 bp and -317/-304 bp, located within the –517/-278 bp region of promoter II. Additionally, two previously identified SF-1 sites and a CRE are present within the –278/-100 bp region. The percentages depict the homology to the consensus sequences.

Figure 5. A C/EBP binding site (-317/-304 bp) is essential for both basal and T47D cell conditioned medium induced activation of promoter II.

TCM induced the activity of –517 bp construct by 5.7 fold as compared with normal mammary epithelial cell conditioned medium (NCM) treatment. Site directed mutagenesis of 5 potentially important cis-acting elements demonstrated that a CRE (-211/-197 bp) and a C/EBP binding site (-317/-304 bp) were essential for TCM induction of promoter II activity. In particular, mutation of the –317/-304 bp C/EBP binding site completely abolished both baseline and TCM-induced activities. Mutations of the two SF-1 sites or another C/EBP binding site (-350/-337 bp) did not affect TCM induction of promoter II activity.

Figure 6. The effects of adipogenic transcription factors, C/EBP α , C/EBP β and C/EBP δ on the activity of –517 bp promoter II construct in HAF

Mammalian expression vectors of C/EBPs were co-transfected into HAF together with the -517 bp promoter II construct. Ectopic expressions of C/EBP β (3.5-fold) and C/EBP α

(2.5-fold) stimulated promoter II activity, whereas C/EBP δ did not have any significant effects.

Figure 7. The effects of T47D conditioned medium on promoter II is mediated by C/EBP β

Panel A: C/EBP β and C/EBP δ bind to the C/EBP site (-317/-304bp) upstream of promoter II in TCM-treated HAF. EMSA was employed using an oligonucleotide probe (-322/303 bp) containing the -317/-304 bp C/EBP binding sequence, nuclear extracts from HAF incubated with or without TCM and supershifting antibodies against C/EBP α , C/EBP β , C/EBP δ and CREB. We identified two specific complexes (1 and 2) as verified by wild type (WT) and mutated (Mut) cold competitors in TCM-treated HAF. Antibodies against C/EBP β or C/EBP δ supershifted complex 1 indicating the presence of C/EBP β and C/EBP δ in this complex. On the other hand, C/EBP α or CREB did not eliminate or supershift any of these complexes. **Panel B:** TCM induced the activity of -517 bp promoter II construct by 6-fold, whereas the addition of C/EBP β to TCM did not increase the promoter II activity any further.

Figure 8. C/EBP β transcripts are induced by T47D cell conditioned medium in HAF.

Human primary adipose fibroblasts (HAF) were treated by media conditioned by normal breast epithelial cells (NCM) or T47D cells (TCM), or left untreated (no treatment) for 48 h. Twenty micrograms total RNA isolated from each sample were then used for northern blot analysis. The 28S RNA fraction was included to demonstrate the presence of comparable amounts total RNA in each lane. TCM profoundly increased the expression

of C/EBP β , whereas the expression patterns of C/EBP α and δ were not altered by TCM treatment.

Table 1. Primers used for site-directed mutagenesis

Mutated Consensus Sequence *	5'-Phosphorylated and Mutagenic Primers *
C/EBP binding site (-350/-337 bp) TTGTTT <u>T</u> GAA <u>A</u> TT→TTGTcccGAgggT	5'-GGG AGA TTG CCT TTT TGT ccc GAA ATT GAT TTG GCT TC-3' 5'-ATT GCC TTT TTG Tcc cGA ggg TGA TTT GGC TTC AAG GG-3'
C/EBP binding site (-317/-304 bp) AGAT <u>T</u> GCCTAA <u>A</u> CA→AgcccGCCTggtCA	5'-TGG CTT CAA GGG AAG AAG ccc GCC TAA ACA AAA CCT GCT G-3' 5'-CAA GGG AAG AAG ccc GCC Tgg tCA AAA CCT GCT GAT GAA G-3'
SF-1 binding site (-263/-251 bp) ATG <u>A</u> GCTTTATTT→ATGgGaaTTATTT	5'- GAC TCC ACC TCT GGA ATG gGa aTT ATT TTC TTA TAA TTT GGC-3'
SF-1 binding site (-136/-124 bp) <u>A</u> GGTCAGAAA→cccTCAGAAA	5'-GGA ACC TGA GAC TCT ACC Acc cTC AGA AAT GCT GCA ATT CAA GC-3'
CRE (-211/-197 bp) TG <u>C</u> ACGTC <u>A</u> CTCT	5'-GGC TTT CAA TTG GGA ATG gAa tTC ACT CTA CCC ACT CAA GGG CA-3'

* Lower cases represent the mutated base pairs

FIGURE 1A

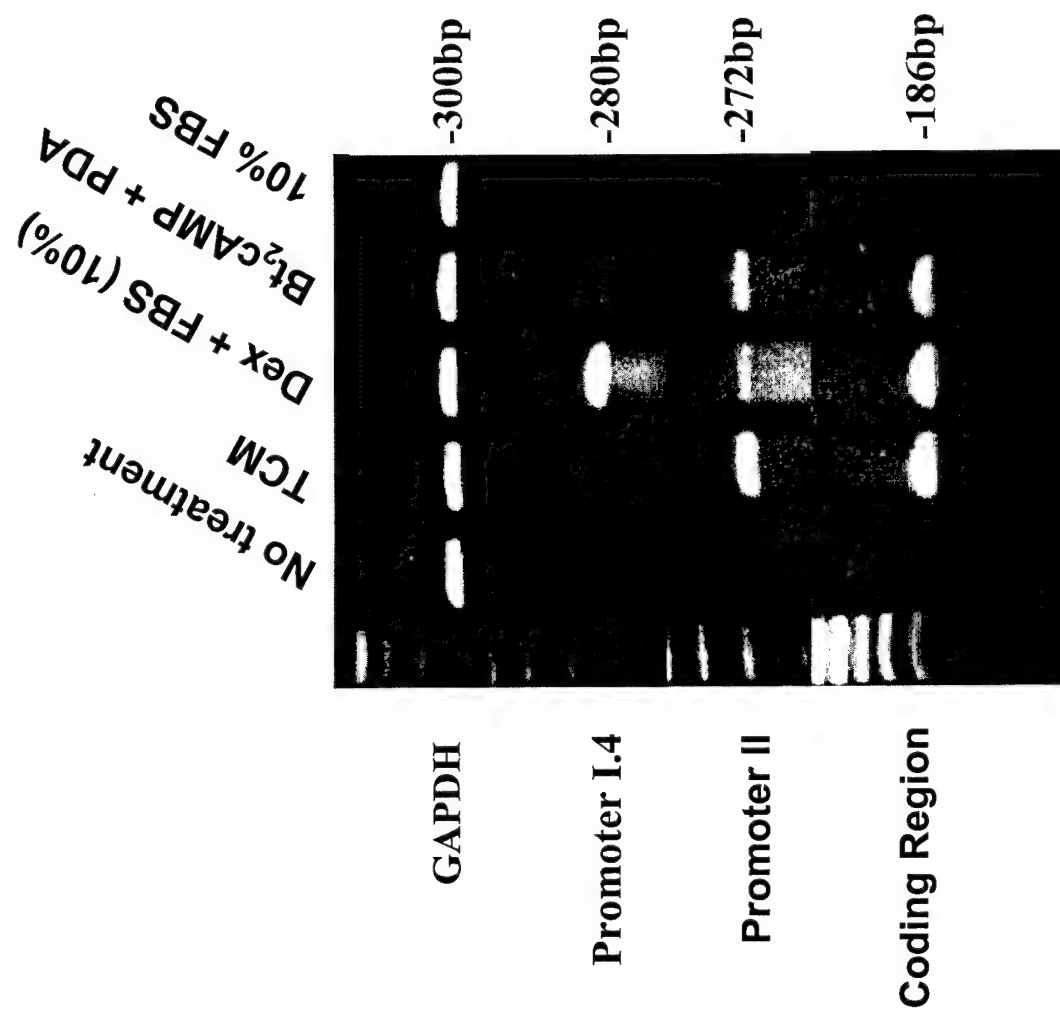


FIGURE 1B

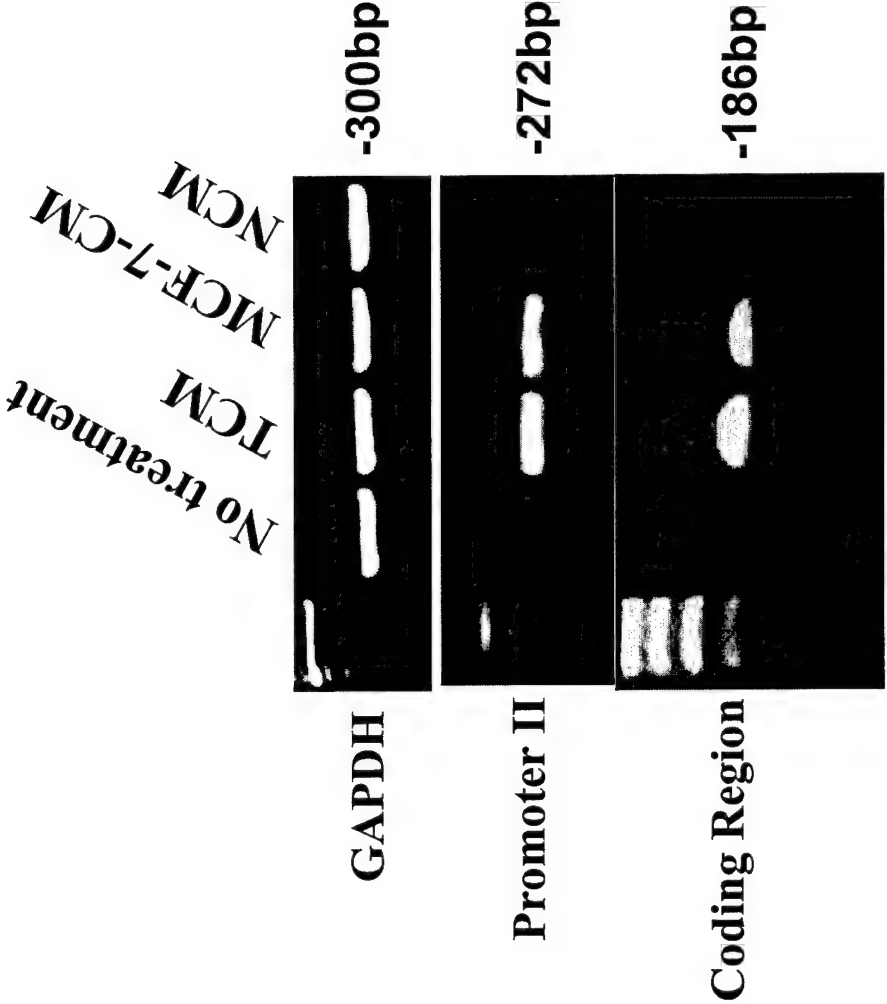


FIGURE 2A

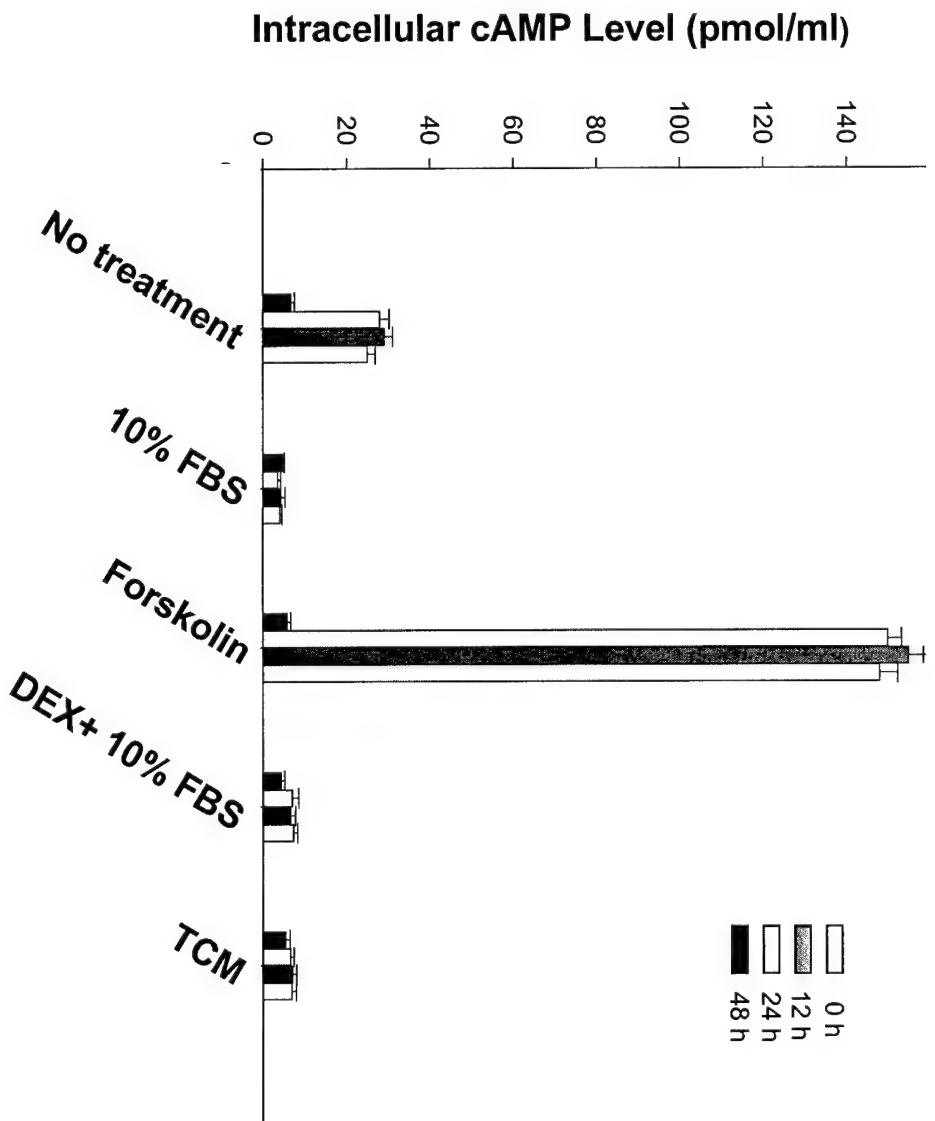


FIGURE 2B

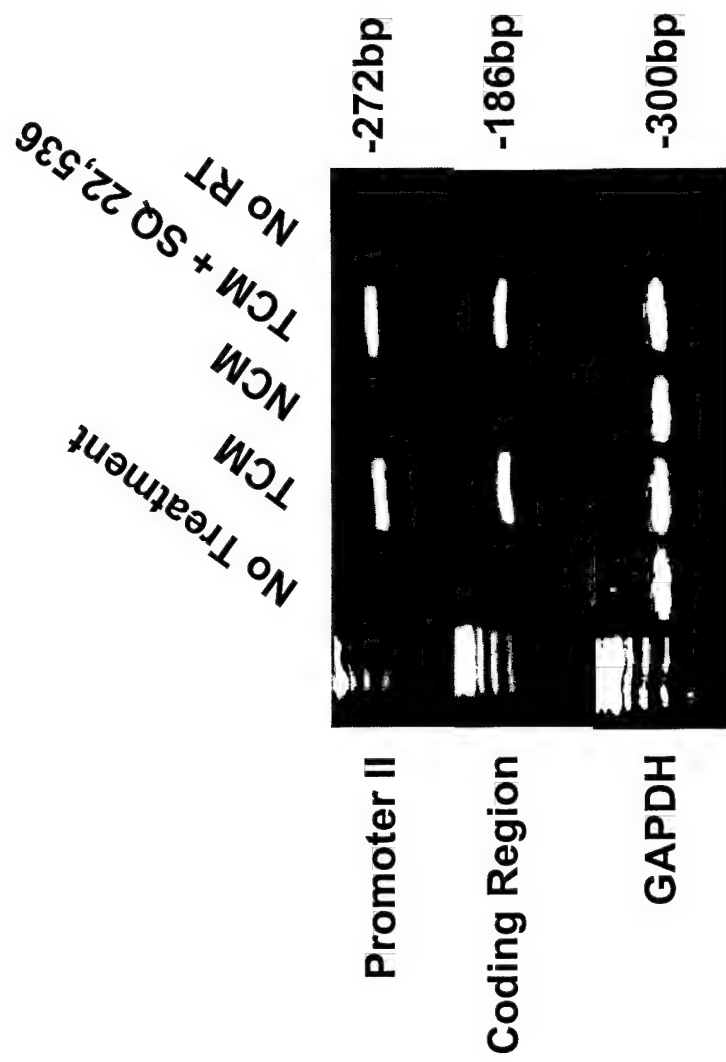
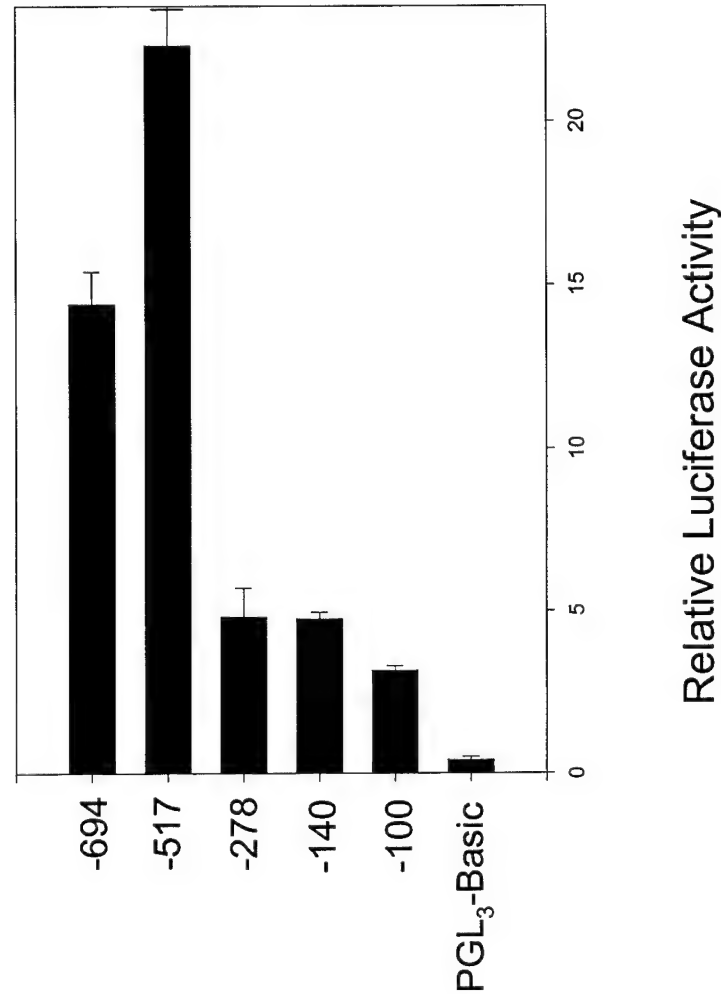


FIGURE 3



-550 CATGGTACAA GAGATTTTATG ATCTTCATTG AAGTCACTAG AGATGGCCTG
-500 AGTGAGTCAC TTTGAATTCA ATAGACAAAC TGATGGAAGG CTCTGAGAAG
-450 ACCTCAACGA TGCCCAAGAA ATGTGTTCTT ACTGTAGAAA CTTACTATTT
-400 TGATCAAAAA AGTCATTTTG GTCAAAAAGG GGAGTTGGGA GATTGCCTTT
CIEBP 1 89% CIEBP 2 98%
-350 TTGTTTTGAA ATTGATTGG CTTCAAGGGA AGAAGATTGC CTAACACAAA
SF-1 85%
-300 CCTGCTGATG AAGTCACAAA ATGACTCCAC CTCCTGGAATG AGCTTTATTT
CRE 86%
-250 TCTTATAAT TGGCAAGAAA TTTGGCTTTC AATTGGGAA TGCACGTCACT
-200 CTACCCACTC AAGGGCAAGA TGATAAGGTT CTATCAGACC AAGCGTCTAA
SF-1 86%
-150 AGGAAACCTGA GACTCTACCA AGGTCAGAAA TGCTGCAATT CAAGCCAAAA
-100 GATCTTTCTT GGGCTTCCTT GTTTTGACTT GTAACCATAA ATTAGTCTTG
TATA
-50 CCTAAATGTC TGATCACATTT ATAAAAACAGT AAGTGAATCT GTACTGTACA

FIGURE 5

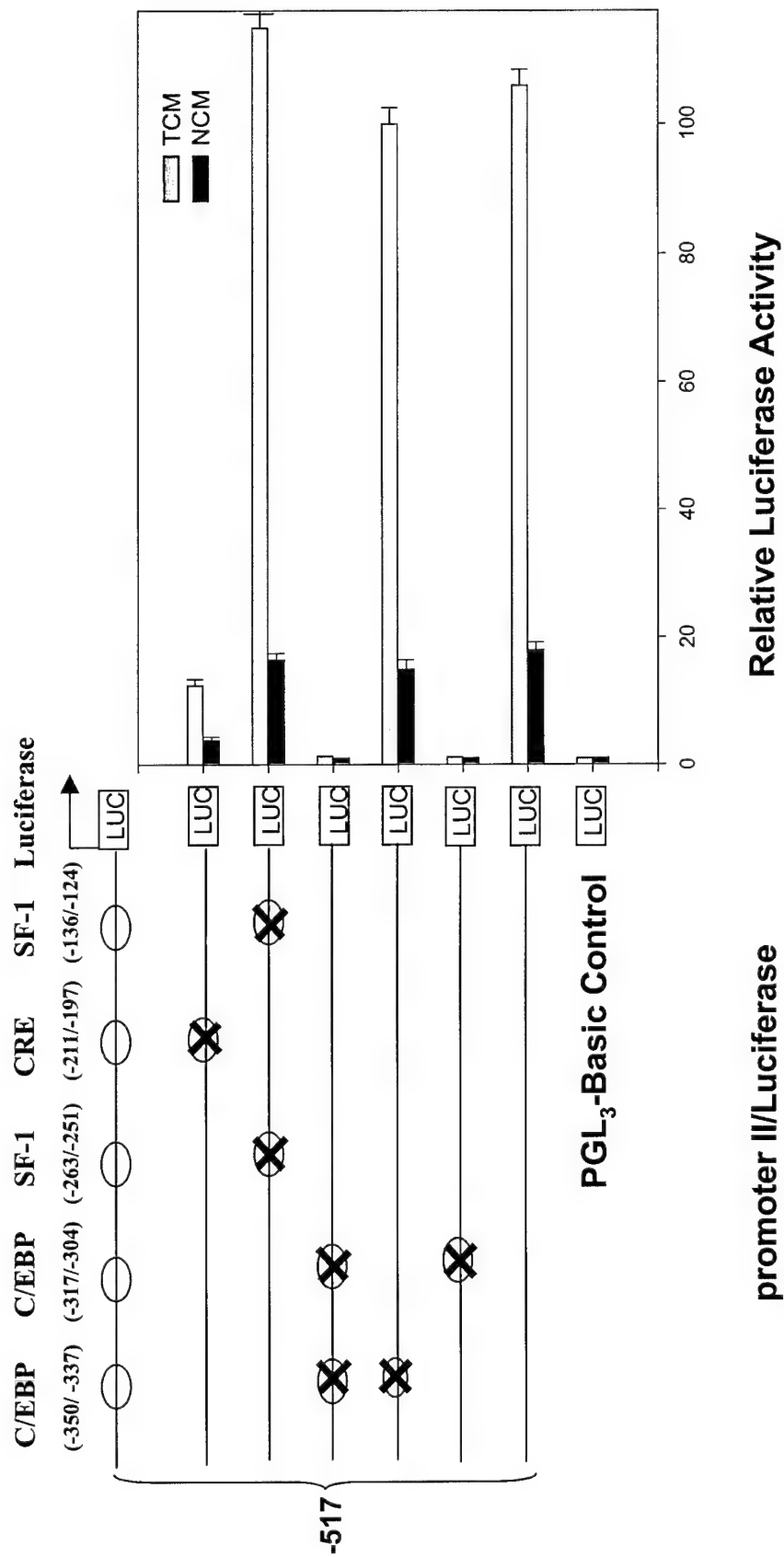


FIGURE 6

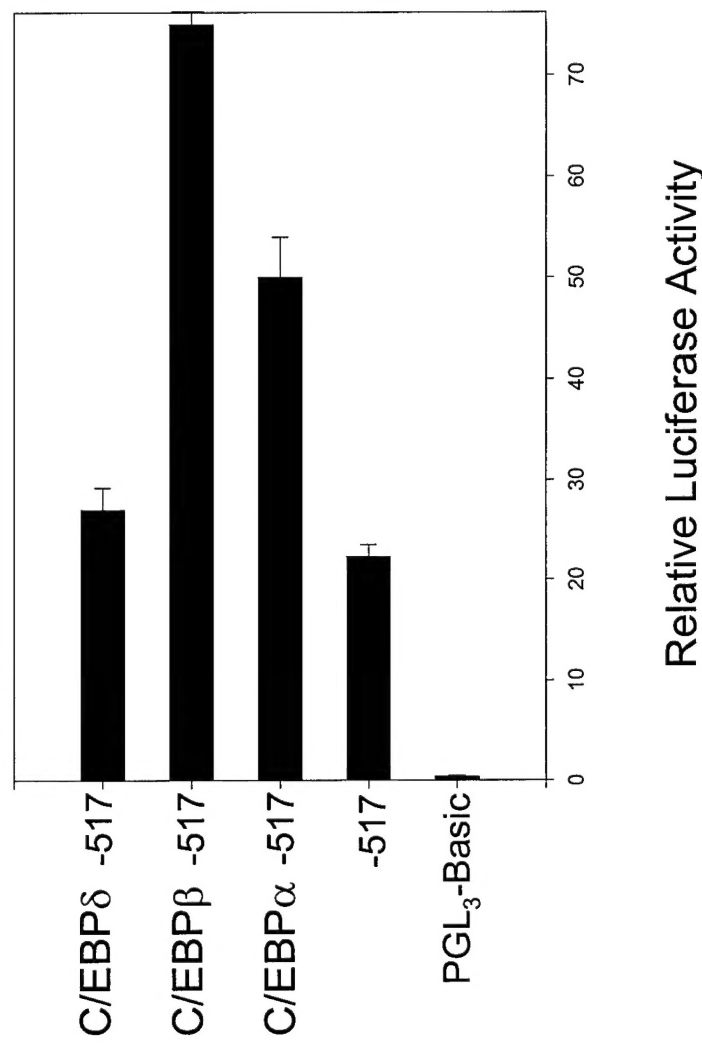


FIGURE 7A

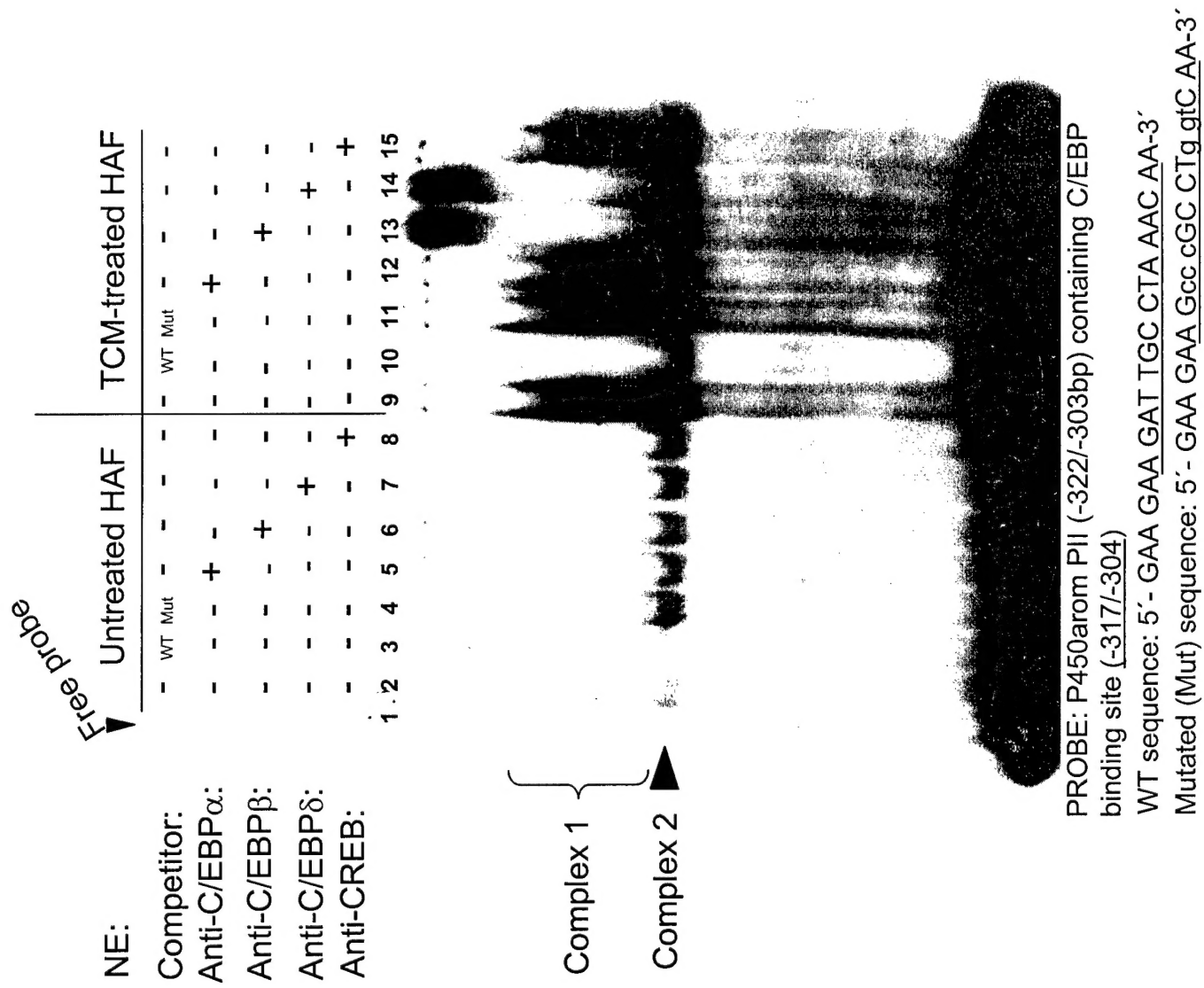


FIGURE 7B

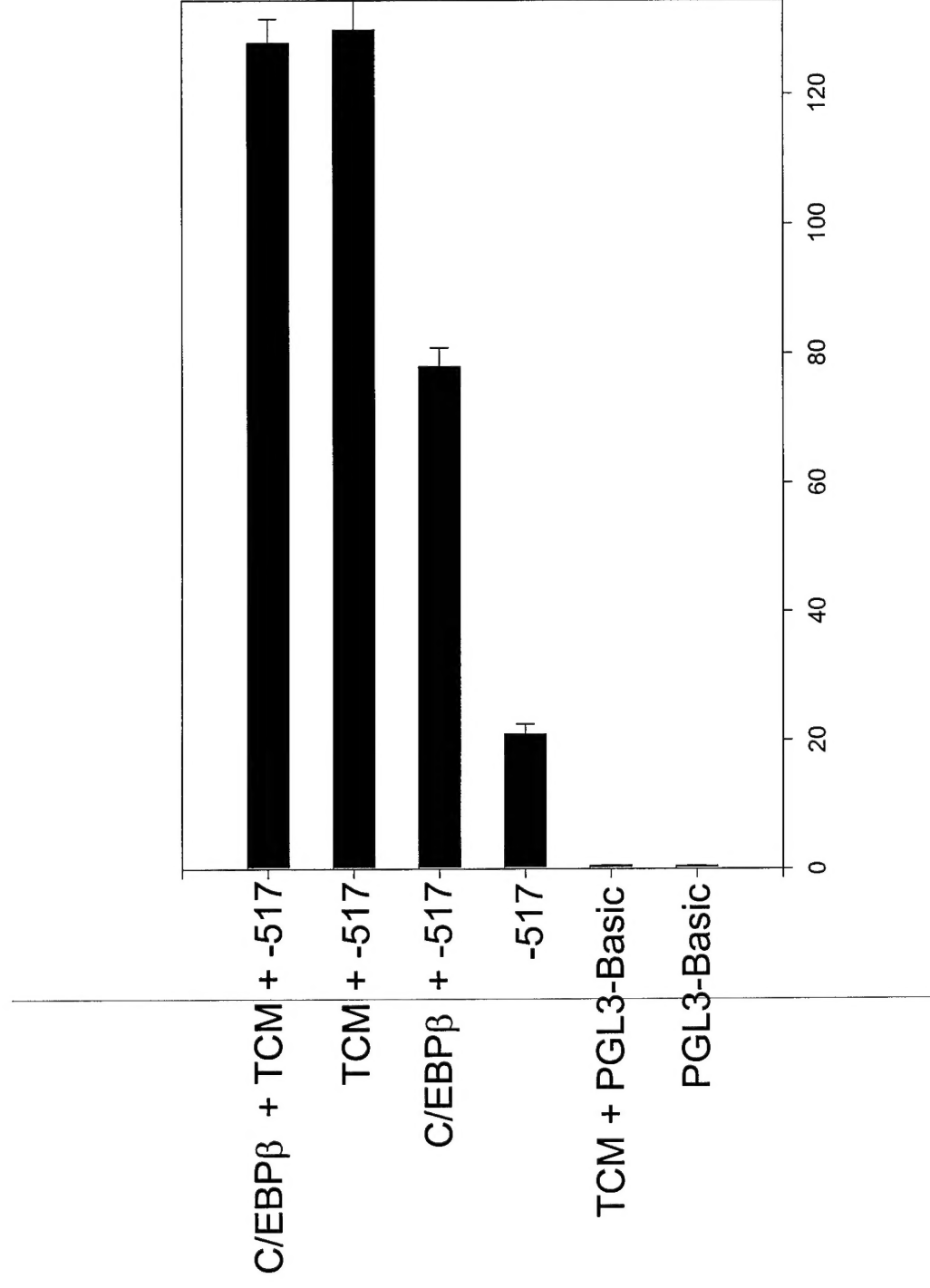


FIGURE 8

